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(71) Applicant: ALFA-LAVAL AGRI INTERNATIONAL AKTIEBOLAG [SE/SE]; P.O. Box 39, S-147 00 Tumba (SE).

(71)(72) Applicant and Inventor: HÖÖK, Magnus [SE/US]; 4235 Oberlin, Houston, TX 77005 (US).

(72) Inventor: McGAVIN, Mary, H.; 495 Lanark Street, Apt. 2 G, Winnipeg, Manitoba R3N 1L7 (CA).

(74) Agent: MUKAI, Robert, G.; Burns, Doane, Swecker & Mathis, Washington & Prince Streets, P.O. Box 1404, Alexandria, VA 22313-1404 (US).

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(54) Title: A BONE SIALOPROTEIN BINDING PROTEIN AS WELL AS ITS PREPARATION

(57) Abstract

The present invention relates to a bone sialoprotein binding protein having a molecular weight of 72 kDa or 60 kDa.

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A BONE SIALOPROTEIN BINDING PROTEIN AS WELL AS ITS PREPARATION

DESCRIPTION

Technical field

The present invention relates to a bone sialoprotein binding protein.

The object of the present invention is to obtain a bone sialoprotein binding protein.

A further object is to obtain a possibility of preparing said protein by chemical synthesis.

Further objects will be apparent from the following description.

Background of the invention

WO-A1-85/05553 discloses bacterial cell surface proteins having fibronectin, fibrinogen, collagen, and/or laminin binding ability. Thereby it is shown that different bacteria have an ability to bind to fibronectin, fibrinogen, collagen, and/or laminin.

Staphylococcus aureus is a major human pathogen and is associated with infections such as bacterial arthritis (4), osteomyelitis (21), and acute infectious endocarditis (19). Staphylococci can also cause pneumonia and tracheobronchitis in immunocompromised patients and patients with cystic fibrosis (20).

The initial step in an infectious disease is often the adhesion and colonization of the host tissue surfaces. A number of bacterial cell surface structures have been identified as mediators of host tissue adherence. Previous studies of Gram negative bacteria, demonstrated that adhesion to tissues is primarily mediated through lectins present on pili or fimbriae (11,16). These lectins, recognize and bind to glycoproteins and glycolipids present on host cell surface. Gram positive bacteria on the other hand appear to promarily adhere to extracellular matrix molecules such as fibronectin via specific cell surface re-

ceptors in what is believed to be protein-protein interactions. Also, some Gram positive bacteria can recognize and bind to extracellular matrix proteins.

The S. aureus fibronectin receptor is probably the best characterized bacterial receptor for an extracellular matrix protein (3,12,15,23). Two very similar and closely spaced Staphylococcal genes coding for fibronectin binding proteins of M_r 210,000 and M_r 165.000 respectively have been identified (23). The fibronectin binding activity is in both proteins located to a segment composed of a 38 amino acid long motif which is repeated three times (12,15, 23). The receptor binds to the N-terminal domain of fibronectin which is not known to contain any carbohydrate and staphylococcal binding to fibronectin therefore probably involves a protein-protein interaction. Staphylococcal cells may also express receptors for thrombospondin, bone sialoprotein, fibrinogen, collagen and vitronectin (3,7,8, 9,25,27). Expression of receptors for matrix proteins with specific tissue distribution may partly explain the tissue tropism of infections caused by various staphylococcal strains. Thus staphylococcal strains isolated from patients with septic arthritis express a collagen receptor and the presence of this receptor appear to be necessary and sufficient for bacteria to adhere to cartilage (26).

In the present study, we screened several S. aureus isolates from different sources for the presence of bacterial proteins capable of binding bone sialoprotein (BSP). BSP is a glycoprotein found only in the bone matrix and dentin (21), and the ability to bind BSP may be a factor which targets S. aureus cells to bone tissue. The data presented here describes a 72 kDa BSP binding protein which is present in many strains. This protein, which is released from bacterial cells by treatment with 1M LiCl and purified by cation exchange chromatography, binds not only BSP but also fibronectin, thrombospondin, vitronectin and fibronogen. Furthermore, the 72 kDa protein has lectin-like

attributes, which may account for its ability to bind a number of matrix glycoproteins. Hence the 72 kDa protein may represent a general adhesin mediating staphylococcal adherence to a variety of extracellular matrices.

MATERIALS AND METHODS

Bacteria and Growth Conditions

The Staphylococcus aureus clinical isolates were from the Department of Orthopedics, Bowman Gray School of Medicine, Winston-Salem, N.C., or the Clinical Pathology Laboratory, School of Medicine, University of Alabama at Birmingham, Birmingham, Alabama (26). These isolates include X50151, 87/8, 87/12 from synovial fluid, F44947 isolated from sputum, M65051 isolated from a soft tissue infection, strain Cowan (25) was isolated from a septic arthritis patient, and 88/4 is an osteomyelitis isolate. Strain 024, an osteomyelitis isolate, was obtained from Dr. C. Rydén, and has been described previously (22). Strain Newman (3) was obtained from the Department of Bacteriology, College of Veterinary Medicine, Uppsala, Sweden and S. aureus strain #574 from the Food and Drug Administration, USA.

Glycerol stocks were made from overnight cultures in tryptic soy broth (TSB, Difco, Detroit, MI) and were stored at -20°C. S. aureus cultures were started by inoculation from glycerol stocks into either TSB or Luria broth (LB, Gibco BRL). After overnight growth at 37°C with agitation, cells were harvested by centrifugation (3600 x g, 15 min) and resuspended in phosphate buffered saline (PBS; 10 mM phosphate, 0,14 M sodium chloride, 0,02% sodium azide, pH 7.4). Cell density was adjusted to 8 x 10^9 cells/ml using a reference standard curve relating optical density at 600 nm to cell number. The cells were kept on ice until use.

Preparation and Iodination of Ligands

The ligands used for this study were provided by the following sources. Bone sialoprotein (BSP), isolated from a rat osteogenic cell line, was a gift from Dr. L. Fisher of the National Institute of Health, Bethesda Maryland. Collagen Type II isolated from chicken sternum was a gift from Dr. R. Mayne, Department of Cell Biology, University of Alabama at Birmingham. Fibronectin (FN) and the 29 kDa N-terminal FN fragment (FN-29) were isolated from porcine plasma as described (2). Human fibrinogen (FBG) was obtained from KabiVitrum, Stockholm, Sweden and further purified by passage through gelatin-Sepharose to remove contaminating FN. Thrombospondin (TSP) purified from fresh human platelets was provided by Dr. J. Murphy-Ullrich, Department of Pathology, University of Alabama at Birmingham. Human vitronectin (VN) was purchased from the Sigma Chemical Company (St. Louis, MO) or Collaborative Biomedical Products (Bedford, MA).

Iodination of ligands was conducted by either the chloramine T method of Hunter (10) or the lactoperoxidase method (17) using enzymobeads (Biorad Corp, Richmond, CA). The estimated specific activities of the iodinated ligands ranged from 2.0×10^6 cpm/µg to 4.4×10^7 cpm/µg.

Binding of Ligands to Bacteria

The binding of radiolabelled ligands to bacterial cells was quantitated essentially as described by Fröman et. al. 1987 (3). Tubes (75 x 12 mm, 4.5 ml, Sarstedt, Newton, NC) were precoated with 5% (wt/vol) bovine serum albumin (BSA, Sigma) in PBS on an end-over-end mixer for a minimum of 1 hour to reduce non-specific binding to the plastic surface. The binding buffer used was either 0.1% (wt/vol) BSA in PBS containing 0.1% Tween-80 (ligand binding buffer I), or 10 mM Hepes pH 7.4, containing 137 mM NaCl, 5 mM KCl, 0.7 mM $MgSO_4$, 1.2 mM $CaCl_2$, and 0.1% BSA (ligand binding buffer II; 22). In either case, 3×10^8 cells were added to 5×10^4 cpm ^{125}I-labelled ligand in a

final volume of 0.5 ml of binding buffer. Tubes containing the incubation samples were rotated end-over-end for 90 min. The reaction was stopped by the addition of 3 ml of ice cold PBS containing 0.1% Tween-80 (PBS/Tween), followed by centrifugation. The supernatant was aspirated, and the radioactivity associated with the bacterial cell pellet was quantitated in a gamma counter (LKB, Turku, Finland). Some binding assays were conducted in the presence of 10 mM EDTA, or 0.2 M mannose.

Solubilization of S. aureus proteins

1. *Solubilization in SDS*

Overnight cultures of S. aureus cells were harvested by centrifugation and bacteria were suspended in PBS to 1/10 of the original volume. The cells were boiled in the presence of 2% SDS and 5% 2-mercaptoethanol for 3 min and loaded onto SDS-PAGE gels.

2. *Lysostaphin digest*

To solubilize S. aureus cell surface proteins, cell were digested with lysostaphin (Sigma) as previously described (22). The residual cells were removed by centrifugation. The solubilized surface proteins recovered in the supernatant were further analyzed.

3. *Trypsin digest*

Overnight cultures of S. aureus cells were harvested by centrifugation (3600 x g, 15 min) and resuspended in 1/10 volume of PBS. The cells were incubated with 25 µg/ml trypsin (Sigma) for 1 h at 37°C. The reaction was stopped by adding 50 µg/ml soybean trypsin inhibitor (Sigma) as described elsewhere (24). The residual cells were collected by centrifugation and used in binding assays.

4. *Lithium chloride extraction*

Overnight bacterial cultures were harvested by centrifugation (3600 x g, 15 min) and resuspended in 1/10 volume of 1M LiCl pH 6.0. The cell suspension was incubated at 45°C for 2 h with gentle agitation. Subsequently, the

cells were removed by centrifugation (3600 x g, 15 min) and the supernatant containing solubilized proteins collected.

Detection of Ligand Binding Components by Western Ligand Blotting

Proteins were separated by SDS-PAGE using gradient gels of 3-15% acrylamide, and the buffer system of Laemmli (14). Proteins were transferred to Immobilon P (Millipore, Bedford, MA) membranes using the Bio-Rad Trans-Blot apparatus, and the transfer buffer of Towbin (28). Additional protein binding sites on the membranes were blocked by incubation for 60 min in PBS containing 3% (wt/vol) BSA. Membranes were then incubated overnight at 4°C with gentle agitation, in a solution containing 1 x 10^5 cpm of ^{125}I-labelled ligand in ligand binding buffer I. Subsequently, the membranes were washed extensively with 0.1% Tween-20 in PBS, air dried and exposed to Fuji RX-100 X-ray film for 4 to 16 h at 4°C.

Purification of Ligand Binding Components

Protein was precipitated from the LiCl extracts by the addition of ammonium sulfate to a final concentration of 60% (wt/vol), followed by gentle stirring overnight at 4°C. The precipitated protein was recovered by centrifugation (15000 x g, 30 min), and the pellet resuspended in a minimal volume of 10 mM Tris-HCl, pH 7.5. In preparation of further purification steps, protein was dialyzed against 3 changes of 10 mM Tris-HCl, pH 7.5 buffer at 4°C.

The dialyzed protein solution was clarified by centrifugation, and subjected to cation exchange chromatography on a Mono-S column (Pharmacia, Uppsala, Sweden) connected to a Pharmacia FPLC system. The column was equilibrated in 10 mM Tris-HCl, pH 7.5, and bound protein was eluted with a linear gradient of up to 1M NaCl in 10 mM Tris-HCl, pH 7.5. The eluate was monitored by the absorbance at 280 nm and positive fractions were analyzed by SDS-PAGE. Ligand binding components were detected by

Western-ligand blotting. Peak fractions containing ligand binding activity were pooled and stored at -20°C.

Haemagglutination of Sheep Red Blood Cells With Purified Protein

The haemagglutination titration was conducted as previously described for the Sophora japonica lectin (16). Serial two-fold dilutions of a 1 mg/ml stock solution of purified protein were made with PBS as the diluent, in a microtiter plate. The final volume of the diluted protein was 100 µl. An equal volume of a 2% suspension of sheep red blood cells (SRBC, Colorado Serum Company, Denver, CO) was added to the protein dilutions. After incubation at room temperature for 1 h, the agglutination was scored. The microtiter round-bottom well containing no protein resulted in the SRBC forming a button whereas agglutination results in a mat of SRBC on the bottom of the microtiter well. The titre was defined as the lowest dilution which caused agglutination of the red blood cells. Inhibition of haemagglutination was tested by addition of serial two-fold dilutions of the following carbohydrates; 0.1 M lactose, 0.1 M mannose, 0.1 M melibiose, 0.05 M L-fucose, 0.1 M N-acetylglucosamine and 0.05 M N-acetylgalactosamine (E-Y Laboratories Inc. San Mateo, CA) in PBS.

Amino Acid Composition Analysis of Purified Protein

Purified protein was analyzed for amino acid composition by the Protein Chemistry Core of the University of Alabama at Birmingham Cancer Center on an Applied Biosystems 420A Derivatizer, a 130A Separation System, and a 920A Data Analysis Module.

RESULTS

Several S. aureus proteins bind BSP

Initial characterization of the binding of BSP to S. aureus strains Newman and #574 indicated that the amounts of radiolabelled ligand bound were not in our hands, affected by the presence of 1.2 M $CaCl_2$, 0.7 M $MgSO_4$

and 10 mM EDTA in the binding buffer as reported in other studies (22). The growth media used to culture bacteria affected the ability particularly of strain #574, to bind BSP. When grown in LB, cells bound as much as 50% more freshly labelled BSP as compared to cells cultured in TSB. Strain Newman bound higher amounts of ^{125}I-BSP, compared to strain #574 and this strain was less affected by the growth media used. Treatment of bacterial cells with trypsin and/or lysostaphin resulted in a two-fold decrease in the amount of ligand bound suggesting that bacterial surface proteins are at least partially responsible for the binding of ^{125}I-BSP. To assess whether the interaction of BSP with the bacterial cells is mediated by specific protein components, whole cell lysates of the various S. aureus isolates were separated on gradient SDS-PAGE gels, and subject to Western ligand blotting with ^{125}I-BSP (Figure 1). The repertoire of BSP binding proteins varied among different strains. All strains analyzed contained a BSP binding protein of approximately 32 kDa and two smaller BSP binding proteins of approximately 18 and 14 kDa. The largest protein species which bound BSP was approximately 85 kDa, and is prevalent in strains F44947, 87/2, #574 and 024. A 72 kDa BSP binding protein was observed in strains X50151, 87/8, 87/2, #574, 024, Cowan and 88/4. A 60 kDa BSP binding protein was predominant in strain Newman, and also observed in strains Cowan, M65051 and 88/4.

Solubilization and purification of a BSP binding protein from S. aureus

Cells of strain #574 were grown overnight in LB and TSB respectively, lysed in SDS and solubilized proteins were separated by SDS-PAGE. The results of these experiments showed that two major proteins with M_r 72,000 and 60,000 respectively, are preferentially expressed by cells grown in LB (Figure 2A, lanes 2 and 5). Western ligand blotting showed the two proteins are major BSP binding proteins (Figure 2B, lane 2). Our studies were therefore

focused on attempts to isolate and characterize these proteins. Extraction of cells with 1M LiCl resulted in the preferential solubilization of the 72 kDa protein from cells grown in LB or TSB (Figure 2A) and Western ligand blotting showed that this protein bound ^{125}I-BSP. The LiCl extract of cells grown in LB also contained three additional BSP binding proteins with apparent molecular weights of 18 kDa, 14 kDa, and 60 kDa respectively.

Since most of the ^{125}I-BSP binding activity is apparently recovered in the LiCl extract, this extraction method was used to solubilize the protein from strain Newman. Solubilized proteins were recovered by ammonium sulfate precipitation and fractionated on a Mono-S cation exchange column fitted on a FPLC system (Figure 3). Absorbance analysis at 280 nm indicated the presence of four peaks which were pooled and analyzed by SDS-PAGE. Fractions pooled from peaks 1 and 2 did not contain detectable amounts of protein. Fractions pooled from peaks 3 and 4 demonstrate a homogeneous 60 kDa protein which eluted between 0.7 M and 0.8 M NaCl. The purified protein was subject to amino acid composition analysis (Table 1) which revealed a high molar percentage of lysine at 11.9%. The level of asparagine and aspartic acid (combined) was considerable at 16.9%. The level of isoleucine and leucine (combined) was 15.8%. The hydrophobic amino acids represent 28.0% of the total amino acids comprising the protein, when alanine (4.8%), valine (6.0%) and phenylalanine (2.4%) are added to the level of isoleucine and leucine.

The 72 kDa protein was purified from the FDA strain #574 in the same manner as described above for strain Newman. The purified proteins were checked for binding activity using the Western ligand blot assay. The purified 60 kDa protein from strain Newman and the purified 72 kDa protein from strain #574 both bound ^{125}I-BSP as demonstrated by Western ligand blotting (Figure 4 . The reason for the observed difference in the apparent molecular

weights of the isolated proteins is unknown. Proteolytic cleavage of the Newman protein during isolation may result in the smaller molecular size.

The purified protein is a general binder of extracellular matrix glycoprotein

The ligand binding specificity of the 72 kDa protein purified from strain #574 was further analyzed. Various radiolabelled matrix glycoproteins including fibronectin, fibrinogen, collagen, vitronectin, and thrombospondin were used to probe Western blots of whole S. aureus #574 cells, LiCl extracts, and the residual extracted cells after growth in both LB and TSB. Remarkably similar patterns were obtained regardless of which ligand was used to probe the membranes although the intensity on the autoradiogram varied considerably. The binding of collagen was less dramatic than with other ligands, particularly vitronectin and thrombospondin. The two higher molecular weight proteins in the LiCl extracts which bound BSP (Figure 2) also appear to bind both ^{125}I-labelled vitronectin and thrombospondin. There is more ligand binding associated with the samples prepared from cells cultured in LB and the major ligand binding protein has a M_r of 72,000 and is solubilized by extraction of bacteria with 1M LiCl. Taken together, these data suggest that the same bacterial protein is capable of binding all the different ligands tested. When the purified 60 kDa protein from strain Newman and the 72 kDa protein from #574 were tested, both proteins bound all radiolabelled ligands demonstrating a broad binding specificity for these proteins. It is noteworthy that the 210 kDa fibronectin (3,23) or the 135 kDa collagen (18,27) receptors could not be detected in Western ligand blots.

If the purified 60/72 kDa protein represents the primary receptor for each of the ligands tested, then this isolated protein would be expected to competitively inhibit ligand binding to intact bacteria. Binding assays were

therefore conducted where increasing amounts of purified protein were added to incubation mixtures containing S. aureus cells and different radiolabelled ligands. The results of these experiments show (Figure 6) that in the case of collagen and the 29 kDa fibronectin fragment, there was no marked change in the amount of iodinated ligand bound to bacterial cells when purified 60/72 kDa protein was added. For all of the other ligands assayed, enhancement of ligand binding to the bacterial cells was observed with increasing amounts of 60/72 kDa protein added. The binding of thrombospondin and vitronectin were increased up to 13 and 15 fold respectively over the amount of radiolabelled ligand bound in the absence of added 60/72 kDa protein. The observed maximal enhancement of BSP, fibronectin, and fibrinogen binding, was 1.5-fold, 2.8-fold and 4.3-fold respectively, above the level observed with no addition of 60/72 kDa protein. The extent of ligand binding enhancement caused by addition of the 60/72 kDa protein varied from one experiment to another and the molecular bases for this effect remains unclear.

The isolated 60/72 kDa protein in an agglutinin

The ability of the purified 60/72 kDa protein to bind several non-related extracellular matrix glycoproteins suggested that this protein may act as a lectin. The protein was therefore tested for the ability to haemagglutinate sheep erythrocytes. The results of these experiments showed that the 60 kDa protein at concentrations of 62 µg/ml and higher agglutinated the SRBC. This interaction could be inhibited by the addition of L-fucose (0.0125 M), D-mannose (0.05 M), and melibiose (0.025 M) but was not affected by lactose (0.1 M), N-acetyl-D-glucosamine (0.1M) or N-acetyl-D-galactosamine (0.1M). Taken together these results suggest that the 60/72 kDa protein may function as an agglutinating lectin and may recognize and bind to carbohydrate structures present in the different matrix glycoproteins.

DISCUSSION

In the study presented in this communication we have used Western ligand blotting to detect ligand binding proteins in Staphylococcus aureus. A surprisingly large number of BSP binding proteins were detected in various S. aureus isolates. The metabolic and structural relationships between these proteins are presently unclear.

Strain Newman and FDA #574 expressed a major BSP binding proteins with apparent molecular weights of 60 kDa and 72 kDa respectively. Both proteins were selectively solubilized by extraction with 1M LiCl and were purified by identical purification protocols. Furthermore, preliminary peptide mapping of these two proteins gave similar fragments (unpublished results MHM and MH). These data suggest that the 60 kDa protein of strain Newman and the 72 kDa protein of strain #574 are closely related. When strain #574 was grown in LB media it bound more ^{125}I-BSP and appeared to express more of the 72 kDa protein compared to cells grown in TSB which suggest that the 72 kDa protein mediates binding of BSP to S. aureus cells.

The enhanced expression of the 72 kDa protein in LB grown cultures may be a consequence of the higher osmolarity of the medium. LB contains a two-fold higher concentration of NaCl than does TSB. Whether osmolarity is the environmental signal governing the expression of this protein has yet to be determined. It is also unclear whether the expression of the 60/72 kDa protein is regulated by the accessory gene regulator (agr)(13).

The 60/72 kDa protein was released from bacteria through extraction with 1M LiCl which suggest that it is not firmly anchored in the cell wall or membrane but associated with the cell surface through an unknown mechanism. The LiCl extract contained only minor amounts of other proteins and purification of the 60/72 kDa protein was a one step procedure on a Mono-S column owing to the highly basic nature of these proteins. The purified protein

had a tendency to self-aggregate in solution perhaps due to the high content of hydrophobic amino acids.

The potential role of the 60/72 kDa protein as a general adhesin is demonstrated by its ability to bind a number of extracellular matrix components. The molecular basis for these interactions are unclear. The 60/72 kDa protein appear to have a lectin-like activity as demonstrated by the ability of the purified protein to haemagglutinate sheep red blood cells in a reaction sensitive to L-fucose, D-mannose and melibiose. Although the haemagglutination reaction is characteristic of lectins, it has also been attributed to lipid-moieties and highly basic proteins (16). Our data indicate that the purified protein is both highly basic and hydrophobic. Therefore these properties could explain the haemagglutination activity of the protein. Further studies are obviously needed to define the mechanism of matrix protein interaction.

The 60/72 kDa protein did not inhibit the binding of radiolabelled ligands to bacterial cells when added to incubation mixtures. Instead, a concentration dependent stimulation of bacterial binding of some ligands was observed. This effect could be explained if the 60/72 kDa protein perhaps through aggregation, acted as a multivalent ligand binder. These large ligand 60/72 kDa aggregates could be formed which subsequently could bind to the cells. Such a mechanism would be facilitated if the ligands contained several binding sites for bacterial proteins.

The data presented describes a cell surface protein on Staphylococcus aureus cells which may act as a general adhesin and bind a number of extracellular matrix components. A general adhesive mechanism has recently been suggested for Treponema denticola with respect to the binding of fibronectin, laminin, and fibrinogen to a 53 kDa and a 72 kDa proteins (5). The Staphylococcus aureus 60/72 kDa protein may represent a similar binding mechanism and

could as such, represent an important virulence determinant.

The present bone sialoprotein binding protein can be used for immunization, whereby the protein, preferably in combination with a fusion protein to create a large antigen to respond to, is injected in dosages causing immunological reaction in the host mammal. Thus the bone sialoprotein binding protein can be used in vaccination of mammals against infections caused by Staphylococci.

Further, the bone sialoprotein binding protein can be used to block an infection in an open skin wound by wound treatment using the bone sialoprotein binding protein in a suspension. Thus the bone sialoprotein binding protein can be used for the treatment of wounds, e.g. for blocking protein receptors, or for immunization (vaccination). In the latter case the host body produces specific antibodies, which can protect against invasion of bacterial strains comprising such a bone sialoprotein binding protein. Hereby the antibodies block the adherence of the bacterial strains to damaged tissue. Treatment of septic arthritis is included as well.

Examples of colonizing of a tissue damage are:
a) colonizing of wounds in skin and connective tissue, which wounds have been caused by a mechanical trauma, chemical damage, and/or thermical damage;
b) colonizing of wounds on mucous membranes, such as in the mouth cavity, or in the mammary glands, urethra, or vagina;
c) colonizing on connective tissue proteins, which have been exposed by a minimal tissue damage (microlesion) in connection with epithelium and endothelium (mastitis, heart valve infection, hip exchange surgery).

When using the present BSP, for the purpose of immunization (vaccination) in mammals, including man, the protein is dispersed in sterile, isotonic saline solution, optionally while adding a pharmaceutically acceptable dispersing agent. Different types of adjuvants can further be

used in order to sustain the release in the tissue, and thus expose the protein or the peptide for a longer time to the immundefense system of a body.

A suitable dosage to obtain immunization is 0,5 to 5 µg of BSP, per kg bodyweight and injection of immunization. In order to obtain a durable immunization, vaccination should be carried out at more than one consecutive occasion with an interval of 1 to 3 weeks, preferably at three occasions.

When using the present BSP, for topical, local administration the protein is dispersed in an isotonic saline solution to a concentration of 25 to 250 µg per ml. The wounds are then treated with such an amount only to obtain a complete wetting of the wound surface. For an average wound thus only a copule of millilitres of solution are used in this way. After treatment using the protein solution the wounds are suitably washed with isotonic saline or another suitable wound treatment solution.

Further the bone sialoprotein binding protein as well as any minimal bone sialoprotein binding site polypeptide, of the present invention can be used to diagnose bacterial infections caused by Staphylococci strains, whereby a bone sialoprotein binding protein of the present invention is immobilized on a solid carrier, such as small latex or Sepharose^{R} beads, whereupon sera containing antibodies are allowed to pass and react with the BSP thus immobilized. The agglutination is then measured by known methods.

Further, the BSP or any polypeptide can be used in an ELISA test (Enzyme Linked Immuno Sorbent Assay; E Engvall, Med. Biol. 55, 193, (1977)). Hereby wells in a polystyrene microtitre plate are coated with the BSP, and incubated over night at 4°C. The plates are then thoroughly washed using PBS containing 0,05% TWEEN 20, and dried. Serial dilution of the patient serum were made in PBS-Tween, were added to the wells, and incubated at 30°C for

1,5 hrs. After rinsing antihuman-IgG conjugated with an enzyme, or an antibovine-IgG conjugated with an enzyme, respectively, horseradishperoxidase or an alkaline phosphatasae, was added to the wells and incubated at 30°C for 1,5 hrs, whereupon when the IgG has been bound thereto, and after rinsing, an enzyme substrate is added, a p-nitrophosphate in case of an alkaline phosphatase, or ortophenylene diamine substrate (OPD) in case a peroxidase has been used, respectively. The plates comprising the wells were thus then rinsed using a citrate buffer containing 0,055% OPD, and 0,005% H_2O_2, and incubated at 30°C for 10 min. Enzyme reaction was stopped by adding a 4N solution of H_2SO_4 to each well. The colour development was measured using a spectrophotometer.

Depending on the type of enzyme substrate used a fluoroscense measurement can be used as well.

Above the term bone sialoprotein binding protein includes a polypeptide sequence as well, which polypeptide sequence forms the minimal bone sialoprotein binding site of the complete protein.

Figure 1 Autoradiogram resulting from Western ligand blotting of S. aureus strains with ^{125}I-BSP

Proteins representing S. aureus cell lysates were separated on 3-15% acrylamide SDS-PAGE gels and transferred to Immulon-P membrane. The membrane was blocked by incubation in a solution of PBS containing 3% BSA (wt/vol) for 60 min. The membrane was then incubated overnight at 4°C in 1 x 10^5 cpm ^{125}I-BSP in ligand binding buffer. The filter was washed in PBS/Tween-20 and air dried. The filter was exposed to Fuji RX-100 X-ray film overnight. Lane 1 represents low molecular weight markers (Biorad). The following S. aureus isolates are indicated by the following lane designations; lane 2 X50151, lane 3 F44947, lane 4 87/8, lane 5 87/12, lane 6 574, lane 7 024, lane 8 Cowan, lane 9 M65051, lane 10 Newman and lane 11 88/4.

Figure 2 Gel electrophoresis and autoradiogram of Western ligand blotting of S. aureus 574 cellular extracts after growth in TSB or LB

The gel electrophoresis (panel A) and the autoradiogram (panel B) were completed as outlined in the legend of Figure 1. Samples in lanes 2, 3 and 4 represent samples from S. aureus cultured in LB. Lane 1 contains prestained molecular weight standards (Biorad). Lane 2 represents S. aureus concentrated cells after boiling in the presence of 2% SDS and 5% 2-mercaptoethanol. Lane 3 contains LiCl released proteins and lane 4 represents the protein profile from S. aureus residual cells after LiCl extraction. The samples in lanes 5, 6 and 7 were prepared from S. aureus cultured in TSB. Lane 5 S. aureus cell lysates after boiling in 2% SDS and 5% 2-mercaptoethanol and Lane 6 contain the LiCl released proteins. Lane 7 represents the residual cell proteins after LiCl extraction. Panel B represents the autoradiogram after ^{125}I-BSP-binding.

Figure 3 FPLC Profile of protein purification and gel electrophoresis of the peak fractions
The chromatography was performed using a Mono-S column and a gradient of Tris-HCl buffered 1 M NaCl as the eluent. The elution profile is shown in panel A. The peak fractions were analyzed by gel electrophoresis and is shown in panel B. Lane 5 represents molecular weight markers. Lanes 1 and 2 represent the protein in peak 1 (the flow through) and the second peak. Lanes 3 and 4 represent the proteins which correspond to peaks 3 and 4 in the FPLC profile which eluted between 0.7 M and 0.8 M NaCl.

Figure 4 Gel electrophoresis and Western ligand blot of purified proteins from S. aureus strain Newman and strain 574 with ^{125}I-BSP
Panel A represents the gel electrophoresis protein profiles after purification and Panel B the resulting autoradiogram of identical samples. Lane 1 (panel A) are high molecular weight markers and in lane 1 (panel B) prestained molecular weight markers. Lanes 2 and 3 correspond to the purified protein observed in the FPLC-peaks 3 and 4 respectively (figure 3) for strain Newman. Lane 4 represents protein isolated from strain 574.

Figure 5 The binding of ^{125}I-ligands to S. aureus 574 cellular protein extracts after culture in TSB or LB
These experiments were conducted as outlined in the legend of Figure 1. The samples in lanes 1 through 7 correspond to the same samples explained in the legend of Figure 2. In panel A the ligand was ^{125}I-fibronectin, panel B ^{125}I-fibrinogen, panel C ^{125}I-collagen, panel D ^{125}I-vitronectin and panel E ^{125}I-thrombospondin.

Figure 6 The effect of purified protein on the ability of S. aureus 574 to bind ^{125}I-ligand

The indicated amount of protein was mixed with 5 x 10^4 cpm of ^{125}I-ligand in an assay volume of 0.5 ml ligand binding buffer and incubated for 30 min on an end-over-end mixer at room temperature. Bacterial cells (8 x 10^8 cells) were added and after 90 min of mixing at room temperature, unbound ligand was diluted by the addition of 3 ml of ice-cold PBS containing 0.1% (v/v) Tween-80. After centrifugation at 1,350 x g for 20 min, the supernatant was aspirated and the radioactivity associated with the bacterial pellet was quantified using a LKB gamma counter. Results are expressed as a percentage of the radioactivity bound by cells in the absence of added protein. The ligands assayed are as shown in the symbols legends.

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Table I

Amino acid composition of protein purified from S. aureus strain Newman

Amino Acid	MOL%
Asparagine/Aspartic acid	16.9
Glutamine/Glutamic acid	6.8
Serine	10.3
Glycine	5.1
Histidine	1.5
Arginine	3.3
Threonine	7.7
Alanine	4.8
Proline	2.2
Tyrosine	6.0
Valine	6.0
Methionine	ND
Cysteine	ND
Isoleucine	7.3
Leucine	7.5
Phenylalanine	2.4
Lysine	11.9
Tryptophan	ND

ND indicates not determined

CLAIMS

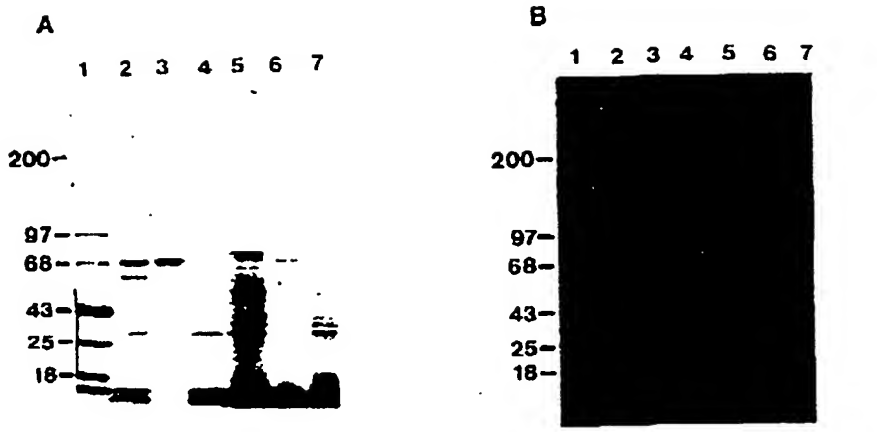
1. A bone sialoprotein binding protein from Staphylococcus, in particular Staph. aureus, which protein further has capability of binding to fibronectin, thrombospondin, vitronectin and fibrinogen, and possesses lectin-like attributes.

2. A bone sialoprotein binding protein according to claim 1, whereby it has an amino acid composition containing 11.9 mol-% lysine, 16.9 mol-% of aspargine and aspartic acid, and 15.8 mol-% of isoleucine and leucine.

3. A bone sialoprotein binding protein according to one or more of the preceeding claims, wherein the protein has a molecular weight of 72 kDa.

4. A bone sialoprotein binding protein according to one or more of claims 1-2, wherein the protein has a molecular weight of 60 kDa.

5. A pharmaceutical preparation containing a therapeutic effective amount of a bone sialoprotein binding protein in accordance with one or more of the preceeding claims together with an inert pharmaceutical carrier or diluent.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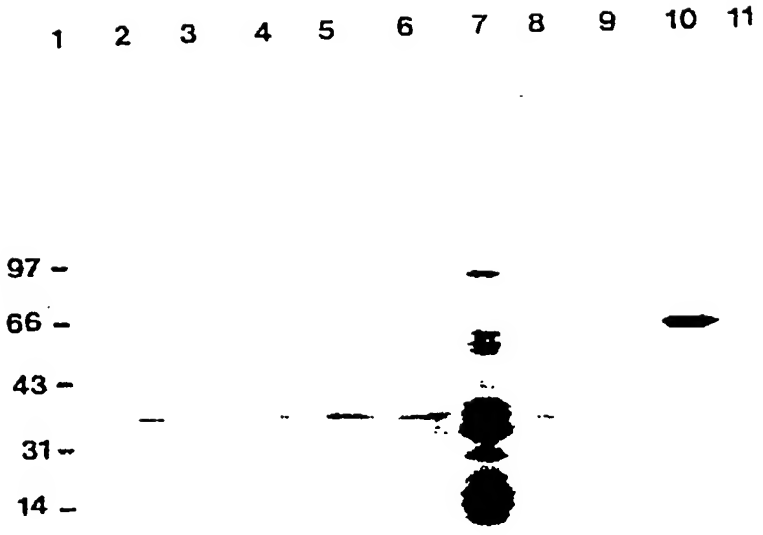
97
68
43
25
18
B
1 2 3 4 5 6 7
200
97
68
43
25
18

Fig 2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66
43
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14

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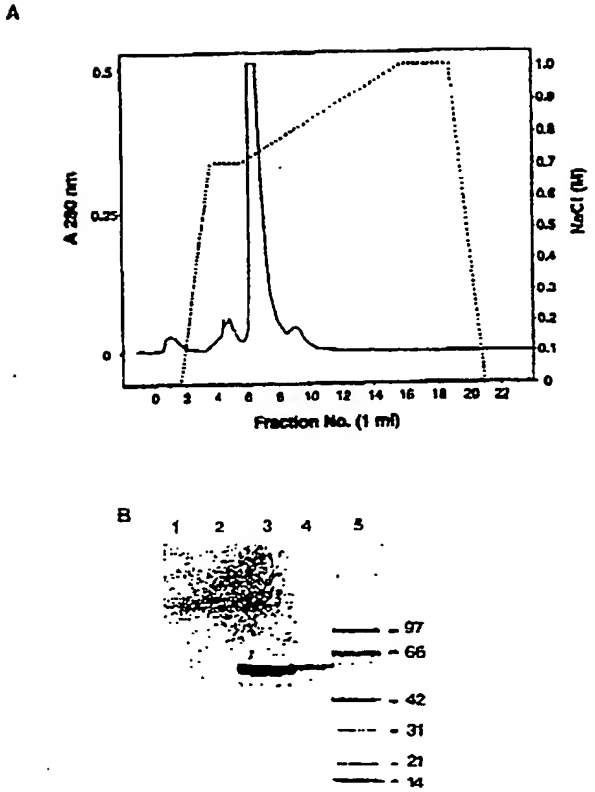
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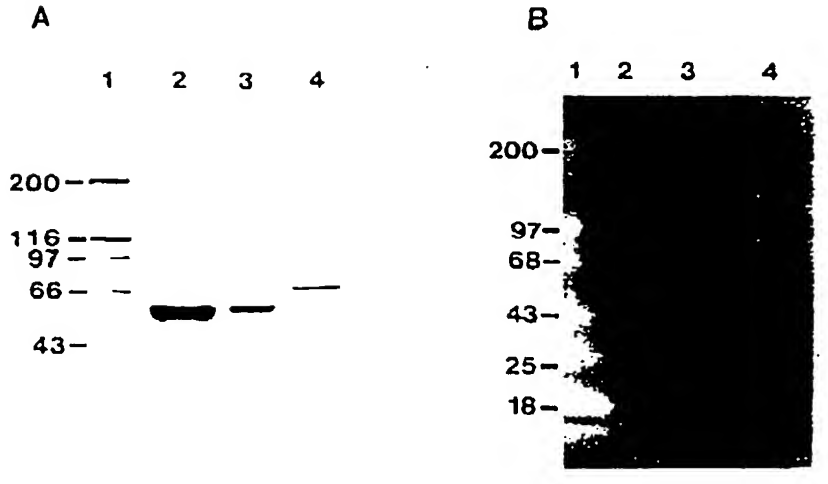
66
42
31
21
14

Fig 3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66
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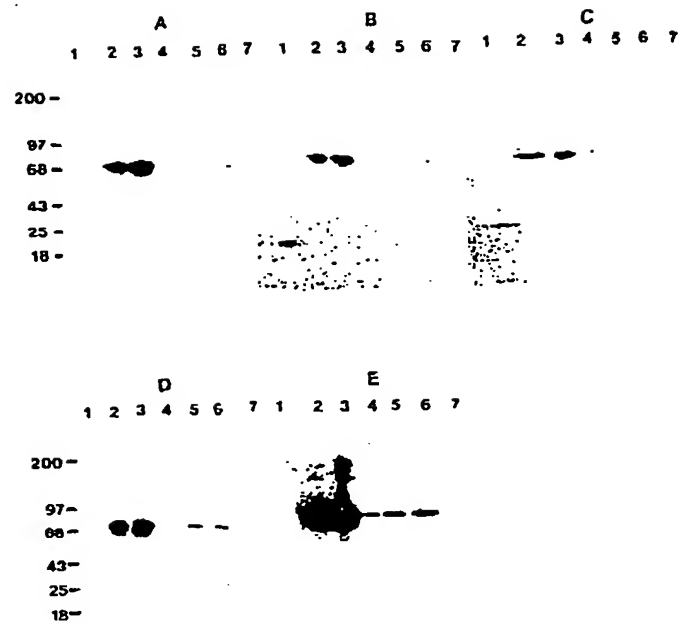
18

Fig 4


A
B
C
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97
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D
E
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Fig 5

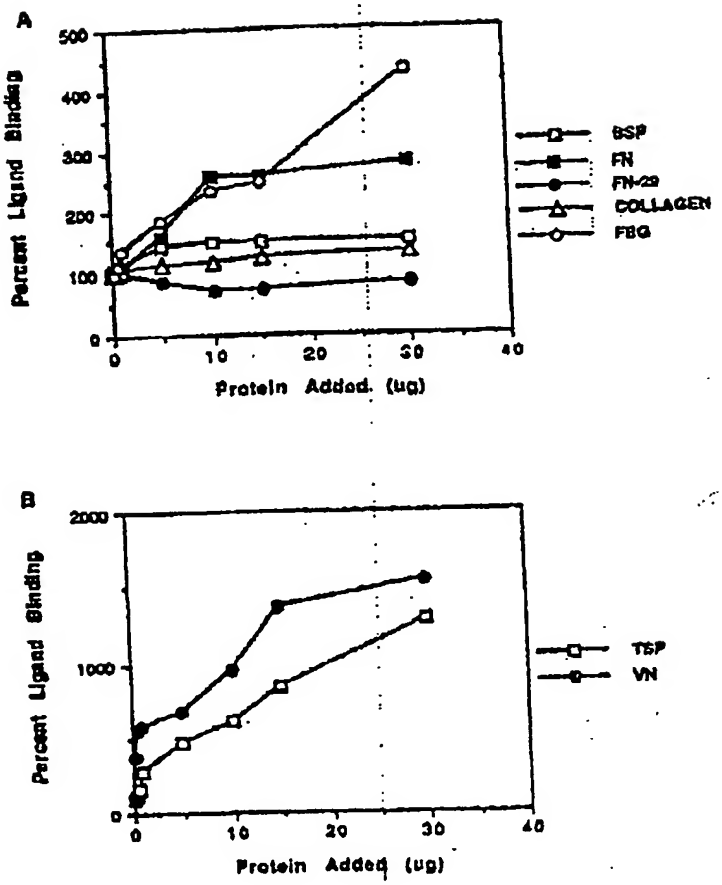
A
Percent Ligand Binding
500
400
300
200
100
0
0
10
20
30
40
Protein Added (ug)
BSP
FN
FN-29
COLLAGEN
FBG
B
Percent Ligand Binding
2000
1000
0
0
10
20
30
40
Protein Added (ug)
TSP
VN

Fig 6

INTERNATIONAL SEARCH REPORT

In .national application No.
PCT/US93/11788

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/00; C07K 3/00, 13/00
US CL :514/21; 530/350, 395, 396, 416, 825
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/21; 530/350, 395, 396, 416, 825

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, MEDLINE, APS, DIALOG, BIOSIS,
Search Terms: Sialoprotein (bone), Staphylococcus (aureus), Fibronectin, Thrombospondin, Vitronectin, Fibrinogen, Lectin.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemical and Biophysical Research Communications, Volume 173, No. 1, issued 30 November 1990, Gotoh et al, "Comparison of Two Phosphoproteins in Chicken Bone and Their Similarities to the Mammalian Bone Proteins: Osteopontin and Bone Sialoprotein II", pages 471-478, see entire document.	1-5
Y	Infection and Immunity, Volume 59, No. 8, issued August 1991, Bremell et al, "Experimental Staphylococcus aureus Arthritis in Mice", pages 2615-2623, especially the abstract.	1-5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 FEBRUARY 1994	Date of mailing of the international search report FEB 28 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer ABDEL A. MOHAMED Telephone No. (703) 308 0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/11788

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Eur. J. Biochem. Volume 184, issued 1989, Ryden et al, "Specific Binding of Bone Sialoprotein to Staphylococcus aureus Isolated from Patients with Osteomyelitis", pages 331-335, especially page 331.	1-5
Y	The Journal of Infectious Diseases, Volume 158, No. 4, issued October 1988, Herrmann et al, "Fibronectin, Fibrinogen, and Laminin Act as Mediators of Adherence of Clinical Staphylococcal Isolates to Foreign Material", pages 693-701, particularly, page 693.	1-5
Y	Infection and Immunity, Volume 60, No. 5, issued May 1992, Haapasalo et al, "Characterization, Cloning, and Binding Properties of the Major 53-Kilodalton Treponema denticola Surface Antigen", pages 2058-2065, especially the abstract and Figure 8.	1-5